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Review Article

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Astrocytes and Microglia as Non-cell Autonomous Players in the Pathogenesis of ALS

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder that leads to a progressive muscle wasting and paralysis. The pathological phenotypes are featured by severe motor neuron death and glial activation in the lumbar spinal cord. Proposed ALS pathogenic mechanisms include glutamate cytotoxicity, inflammatory pathway, oxidative stress, and protein aggregation. However, the exact mechanisms of ALS pathogenesis are not fully understood yet. Recently, a growing body of evidence provides a novel insight on the importance of glial cells in relation to the motor neuronal damage via the non-cell autonomous pathway. Accordingly, the aim of the current paper is to overview the role of astrocytes and microglia in the pathogenesis of ALS and to better understand the disease mechanism of ALS.

Key words: amyotrophic lateral sclerosis, astrocyte, microglia, motor neuron, non-cell autonomous toxicity

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder with a prevalence of 2~3 per 100,000 people and is generally fatal within a few years of disease onset. Affected motor neurons in the brain stem, spinal cord, and motor cortex undergo significant loss, and it eventually causes progressive muscle wasting and paralysis in ALS patients. ALS was initially reported by Dr. Jean-Martin Charcot, a French neurologist, in 1869 [1]. Since Charcot's initial reporting, ALS received international attention when Lou Gehrig, a baseball player of the New York Yankees (Bronx, NY, USA), retired from baseball after being diagnosed with ALS in 1939. For this reason ALS has also been referred as 'Lou Gehrig's disease'. Interestingly, Gulf War veterans have a significantly increased risk (above two fold) of developing ALS [2]. Evidence has shown that the incidence of ALS has risen in recent years and it is reasonable to expect that it will continue to rise in the future. Most cases of ALS occur sporadically, but about 5~10% of ALS cases are familial ALS (FALS). In FALS, more than 90 mutations are found in *superoxide dismutase 1* (*SOD1*) gene [3-6]. In addition, other mutations in *FUS/TLS* and *TDP-43* genes have been known in ALS. Recently, a hexanucleotide repeat expansion of the *C9orf72* gene has been identified as the most common cause of FALS discovered to date [7-15]. Given that mutations of the important cellular antioxidant enzyme SOD1 are a cause of FALS, it has well been proposed that oxidative stress plays a key role in the disease pathogenesis. Indeed oxidative damage and gliogenesis in both postmortem human FALS and sporadic

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ALS (SALS) tissue and in transgenic (mutant SOD1 (G93A)) ALS animal models have been documented [16, 17]. Abnormal regulation of glutamate-dependent excitatory signal has also been identified in ALS suggesting that excessive synaptic glutamate and oxidative stress trigger motor neuronal damage. Moreover, altered calcium homeostasis, mitochondrial dysfunction, protein aggregation, cytoskeletal disruption, apoptosis, and inflammation are associated with motor neuronal damage and cell death [5, 18]. Current medical care for both FALS and SALS focuses on symptom management. Supportive care can help control symptoms and make ALS more manageable for patients and their families, but this care does not significantly improve the disease progression. Even, to date, there are no effective drug therapies that slow the relentless progression of ALS [19-21]. In this regard, the better understanding of pathogenic mechanism of ALS may enhance the possibility for ameliorating the disease onset and progression. In this review, we focus on how non-neuronal cells are associated with the pathogenesis of ALS.

WHAT IS NON-CELL AUTONOMOUS TOXICITY?

In the past when scientists had focused on the study of neuronal function and activity, the events related to neuronal damage and cell death were only investigated from a narrow viewpoint. This view was based on the notion that neurons are damaged due to the dysfunction and deregulation by themselves (so called cell autonomous pathway), and this damage was not related to the dysfunction of any other cell types. As time went by, the view and knowledge of scientists on the mechanisms of neuronal damage have more evolved and advanced. Importantly, a growing body of evidence have proven that non-neuronal cells such as astrocytes, microglia, and oligodendrocytes directly contribute to the motor neuronal damage and cell death (so called non-cell autonomous pathway) in ALS including other neurodegenerative diseases. Indeed, the disease onset and progression is modulated via non-cell autonomous pathway in transgenic ALS [mutant SOD1 (G93A)] mice [18]. The mutant SOD1 expression within motor neurons initiates a damage process and drives the disease onset. In parallel, activation of astrocytes and microglia by mutant SOD1 markedly exacerbates the disease progression while motor neuronal mutant SOD1 has little influence on the progression of ALS. Thus, the paradigm of the non-cell autonomous toxicity has been determined and proven in several experimental conditions of ALS [22, 23].

HOW DO ASTROCYTES MIND MOTOR NEURONS?

A major pathological feature of ALS is the generation and migration of new cells, specifically astrocytes, within and around damaged regions of the spinal cord [24]. Astrocytes respond to cellular stresses by proliferating and adopting a reactive phenotype characterized by the development of long and thick processes with an increased content of glial fibrillary acidic protein (GFAP). Interestingly, a similar increase in GFAP immunoreactivity was found when cultured primary spinal cord astrocytes were exposed to oxidative stress, suggesting that such morphological changes may be triggered by stress signals [24]. It seems likely that epigenetic alterations induced by mutant SOD1 (mtSOD1) and other pathological stresses are involved in the transformation of astrocytes to a neurotoxic reactive phenotype. In this scenario, non-cell autonomous cell death of motor neurons in ALS could result from either a loss of normal astrocytic support and/or the secretion of neurotoxic cytokines. Several studies have proven this idea as following: co-culture of astrocytes expressing mtSOD1 (G93A) or exposure to conditioned medium derived from astrocytes expressing mtSOD1 (G93A) damages both primary motor neurons and embryonic stem cell-derived motor neurons [25, 26]. Previous studies have suggested that cytokines and other toxic factors released from SOD1(G93A) astrocytes may trigger motor neuronal damage [27-30]. For example, *in vitro* studies by Ferraiuolo et al. (2011) show that SOD1(G93A) astrocytes are toxic to normal motor neurons by reducing metabolic support from lactate release and activating pro-nerve growth factor-p75 receptor signaling pathway [27]. Interestingly, SOD1 (G93A) astrocytes specifically express NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome complexed with the NLR protein NLRP3, the adaptor ASC and pro-caspase 1, indicating that astrocytes mediate the neuroinflammation in ALS [28]. Moreover, transforming growth factor-β1 (TGF-β1) is increased in SOD1(G93A) astrocytes, and astrocyte-specific overexpression of TGF-β1 in SOD1(G93A) mice accelerates disease progression in a non-cell-autonomous manner [29]. On the other hand, the elevation of Bid, a BCL-2 family protein, in SOD1(G93A) astrocytes suggests that Bid activation may contribute to astrocyte activation and motor neuronal damage in ALS [30]. In this study, Bid is necessary for activating nuclear factor-κB in astrocytes to mediate pro-inflammatory stimuli, which represents that Bid is not directly toxic to motor neuron but indirectly modulates the astrocyte-dependent non-cell autonomous toxicity. Together, it has been successfully proven that astrocytic cytokines and toxin could determine disease progression and are critical to the pathogenesis of ALS.

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Excitatory amino acid transporter-2 (EAAT2) is known as a typical glial glutamate transporter that uptakes neurotransmitters glutamate and aspartate from the synaptic cleft [31]. It is believed that EAAT2 uptakes more than 90% of glutamate into glia. In normal condition, astrocytes uptake glutamate and turn it into glutamine, and nourish motor neurons by supplying them as energy source. However, when astrocytes become reactive, the expression of *EAAT2* gene is decreased and subsequently an excess amount of extracellular synaptic glutamate may lead to excitocytotoxicity in motor neurons in the spinal cord of ALS. Indeed, as the dysfunction of EAAT2 is implicated in ALS, the level of EAAT2 is reduced in the motor cortex and spinal cord of ALS patients [32]. Moreover, the decrease of EAAT2 activity impairs motor neuron survival in mouse models of ALS [33]. Otherwise, not only does chemical induction of EAAT2 activity improve motor neuron survival in an *in vitro* model of chronic excitotoxicity but it also extends the survival of transgenic ALS mice [34, 35]. When EAAT2 transgenic mice is crossed with mutant SOD1 (G93A) mice, it shows a significant delay in motor symptom such as grip strength decline but not in the onset of paralysis [36]. Interestingly, Foran et al., (2011) reports that sumoylated carboxy-terminal fragment of EAAT2 (CTE-SUMO1) is accumulated in the nucleus of astrocytes in the spinal cord of SOD1(G93A) mice [37]. The expression of CTE-SUMO1 in spinal cord astrocytes produces extrinsic toxicity by inducing caspase-3 activation and impairs axonal growth of motor neurons in a co-culture system. This study provides an unconventional role of EAAT2 in that EAAT2 participates in motor neuron degeneration through the direct cytotoxic effect of its truncated peptide but not through the activity of glutamate transporter. All together, growing evidence supports that regulation of EAAT2 activity accounts for motor neuronal survival and death in ALS via a non-cell autonomous pathway.

In comparison to the astrocytic phenotype in ALS, different astrocytic behaviors in relation to the excitotoxicity may be derived due to either the different damage region of CNS (brain versus spinal cord) or the different stress stimuli (bolus excitotoxicity versus chronic oxidative stress). For instance, GFAP-positive astrocytes appear extensively around the damage sites 7 days after injection of N-ethyl-D-aspartic acid (NMDA) while EAAT2- and GFAP-positive astrocytes disappear in a kainic acid (KA)-injected cortical region of the brain [38]. This study shows that two excitotoxic injury models exhibit quite different pattern of astrocyte behaviors such as astrogliogenesis versus astrocyte loss that are distinguished from the pathology of ALS. Accordingly, it will be challenging to pursue how the difference of region or stress stimuli concerts and affects astrocyte behaviors in future studies.

HOW ARE ASTROCYTES ADAPTED TO ENVIRONMENTAL STRESSES AND WHAT ARE THE SURVIVAL MECHANISMS OF ASTROCYTES UNDER ALS CONDITION?

Our group has previously addressed this question using primary astrocytes from the spinal cord of wild type (WT) and ALS transgenic [mutant SOD1 (G93A)] mice. Our study shows that astrocyte survival is correlated with the elevation of Ets-2 transcription factor and with *Bcl-xL* expression [39]. The transcriptional activation of *Bcl-xL* by Ets-2 compensates oxidative stress by preventing astrocytes from apoptotic or necrotic cell death during the pathogenesis of ALS. Because we observed that motor neurons do not induce Bcl-xL in response to oxidative stress, we suggest that molecular mechanisms of Ets-2-mediated and Bcl-xL-dependent survival pathways may vary among different cell types [39]. Then why are motor neurons of ALS not rescued by the surviving astrocytes? We propose a plausible mechanism that the Ets-2 and Bcl-xL pathway improves astrocyte survival but it occurs too late to prevent earlier motor neuronal damage, or perhaps survived reactive astrocytes release toxic molecules to propagate motor neuron damage (Fig. 1). However, whether this might be expected to occur at an earlier stage, before astrocyte activation is reached its threshold, remains to be further investigated.

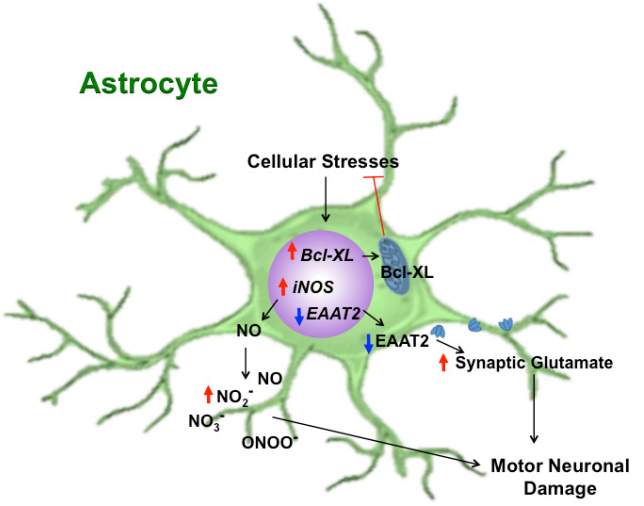

Fig. 1. Astrocytes are associated with non-cell autonomous motor neuronal damage in ALS. For example, cellular stresses elevate *Bcl-xl* gene expression in astrocytes, and the increase level of mitochondrial Bcl-xl prevents oxidative damage in astrocytes under ALS condition. Meanwhile, the increased *iNOS/NOS2* expression and the decreased *EAAT2/GLT-1/SLC1A2* expression in astrocytes lead to increased NO release and decreased glutamate uptake in the synaptic cleft of spinal cord. Consequently, elevation of glutamate and NO triggers motor neuronal damage and cell death via non-cell autonomous pathway.

Oxidative stress due to the mutation of SOD1 is highly implicated in the pathogenesis of ALS. Not only does superoxide anion (O_2^-) lead to cellular damage including oxidation of DNA and protein and lipid peroxidation but nitric oxide (NO) is also thought to play a key pathogenic role in ALS [40]. Motor neurons are particularly vulnerable to oxidative stress in ALS which is a phenomena attributed to a low level of antioxidant enzymes and a high content of easily oxidized substrates [5, 24, 40]. NO is synthesized by NO synthases (NOSs) from arginine, which is a rate-limiting factor for NO production. We have reported that neuronal NOS (nNOS)-positive motor neurons are depleted while inducible NOS (iNOS)-positive reactive astrocytes are increased in ALS transgenic [mutant SOD1 (G93A)] mice [41]. The expression of *iNOS*/NOS2 was correlated with the increases of astrocyte activation and NO levels while *nNOS/NOS1* expression was decreased in ALS transgenic [mutant SOD1 (G93A)] mice. The high levels of NO interact with superoxide and form highly toxic peroxynitrite. Consistent with findings previously reported by Przedborski and colleagues, increased levels of NO may further exacerbate oxidative stress and trigger motor neuron death [40-42]. As similar to ALS transgenic mice, accumulation of 8-hydroxy-2-deoxyguanosine, a marker of oxidative DNA damage, and elevated levels of peroxinitration damage (production of nitrotyrosine residues by covalent interactions of NO) have also been found in human ALS [43-46]. These data support a prominent role of oxidative stress derived from reactive astrocytes during the pathogenesis of ALS (Fig. 1).

IS MICROGLIAL ACTIVATION A GOOD SIGN OR A BAD SIGN TO MOTOR NEURONS?

Despite its controversy, microglia are also known to be linked to motor neuronal damage and the pathogenesis of ALS via the non-cell autonomous pathway [22, 47]. Interestingly, deletion of NF-κB signaling in microglia rescues motor neurons from microglial-mediated death *in vitro* and extended survival in ALS mice by deregulating proinflammatory microglial activation. In contrast, selective NF-κB inhibition in ALS astrocytes was not sufficient to rescue motor neuron death [48]. In this context, the microglia-mediated damage and toxicity to motor neurons are driven through the diversity of death mechanisms. Using the mice carrying deletable mutant SOD1 transgene by the action of Cre recombinase, Yamanaka and Yamashita have shown that diminishing mutant SOD1 toxicity within microglia significantly slowed the disease progression of ALS. This finding suggests that, in part, microglia contribute to neurodegenerative process of ALS [49].

On the other hand, in order to examine whether proliferating microglia leads to motor neuron degeneration in ALS mice, Gowing et al. (2008) generated double transgenic mice with CD11b-TK(mut-30) and mutant SOD1(G93A) in which a 50% reactive microglia is specifically reduced in the lumbar spinal cord [50]. Unexpectedly, reduction of reactive microglia had no effect on the degeneration of motor neuron. This study implies that proliferating microglia-expressing mutant SOD1 (G93A) does not play a pivotal role in triggering neuronal damage in an animal model of ALS. This study raises a question regarding whether different stages of microglia are involved in different modes of action for protecting versus being involved in the damaging of motor neurons through yet unidentified mechanisms. We suggest that future studies are necessary to uncover the precise action mechanism behind the obscure role of microglia in ALS.

WHY IS IT NOT CONSISTENT TO OBSERVE THE ROLE OF MICROGLIA IN THE NEURODEGENERATIVE PROCESS OF ALS?

Is microglia activation beneficial or disadvantageous to motor neurons? Microglia function is necessary for surveilancing the condition of motor neurons and for restoring tissue injury in response to acute and reversible stress: microglia are beneficial before the threshold limit reached. However, constitutive activation of microglia by a chronic and irreversible stress such as ALS stress may transform them as a non-cell autonomous player to be toxic to motor neurons: microglia are disadvantageous after they become fully activated.

We have previously found that the expression of c-Ret is altered in motor neurons of the lumbar spinal cord in ALS transgenic [mutant SOD1 (G93A)] mice and ALS [mutant SOD1 (G85R) and (G93A)] motor neuronal cell lines [51]. c-Ret oncoprotein is a protein kinase receptor and responds to glial cell line-derived neurotrophic factor (GDNF). c-Ret-mediated signal transduction is important to maintain cellular activity and survival function. Notably, the levels of non-phosphorylated and phosphorylated c-Ret were markedly elevated in active microglia of the lumbar spinal cord of ALS mice in an age-dependent manner. Our findings suggest that ALS stress-induced expression of c-Ret in microglia may trigger non-cell autonomous toxic signals and exacerbate damage responses in motor neurons by disturbing the GDNF signaling pathway in motor neurons [51]. Our previous study does not provide a direct evidence that microglia contribute to non-cell autonomous motor neuronal damage in ALS. However, based on our findings, we suggest an indirect contribution of microglia to motor neuronal damage. For instance, the increased level of c-Ret in microglia elevates interaction with GDNF. As a result, the c-Ret and GDNF interaction promotes the survival of

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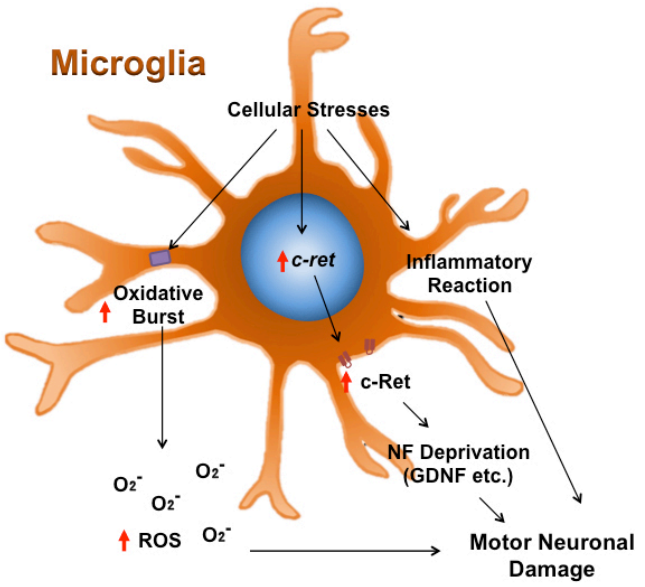

Fig. 2. Microglia may contribute to non-cell autonomous motor neuronal damage in ALS. Under ALS condition, cellular stresses elevate *c-Ret* gene expression in microglia but not in motor neurons. The increased level of c-Ret in microglia interacts with neurotrophic factors (NFs) such as glia derived neurotrophic factor (GDNF). The c-Ret and GDNF interaction in microglia improves their survival under ALS condition, whereas the deprivation of NFs in the niche of spinal cord by activated microglia may result in motor neuronal damage.

microglia whereas the subsequent deprivation of NFs by activated microglia in the niche of spinal cord may lead to motor neuronal damage (Fig. 2).

CONCLUSIONS

A vicious cycle of ALS stresses transforms astrocytes and microglia from Dr. Jekyll to Mr. Hyde

In the pathogenesis of ALS, non-motor neuronal cells such as astrocytes and microglia undergo a series of molecular and cellular changes in that these cells become unprofitable to motor neurons, leading to irrecoverable neurodegeneration. The mechanism of non-cell autonomous motor neuron death is closely associated with the pathophysiological change in ALS that is apparently distinguished from cell autonomous pathway.

Neuroinflammation is now identified as a key contributor to motor neuron damage in ALS [52-54]. Reactive astrocytes and microglia are triggers of neuroinflammation that accelerate disease progression [55, 56] which is further exacerbated by ongoing neuronal injury [53]. Inflammatory cytokines released by astrocytes and microglia may facilitate glutamate excitotoxicity thereby linking neuroinflammation and excitotoxic death [18, 57, 58].

Taken together, previous findings suggest that the molecular and cellular adaptation between astrocytes, microglia, and motor neurons may be differently modulated by epigenetic components upon ALS stresses. In this paradigm, due to chronic oxidative stress or other irreversible mechanisms, a critical threshold limit is reached and that reactive astrocytes and microglia trigger the pathological processes that subsequently lead to a non-cell autonomous death of motor neurons in ALS. This idea suggests that future therapeutic strategy for the treatment of ALS should be aimed at specific interception of pro-oxidant and pro-death signals in a cell-type specific manner [59-62].

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